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14. ABSTRACT

We previously demonstrated that stromal TGF-beta signaling induced the expression of several AR targets as well as MAPK4 in PCa LNCaP cells, and that MAPK4 induced ligand-independent AR activation in PCa cells. Therefore, we proposed to use in vitro PCa/stroma co-culture models and in vivo xenograft models to test our hypothesis on stromal TGF-beta signaling inducing MAPK4 for androgen-independent AR activation in PCa as a direct mechanism for CRPC relapse. In this second year, we have met some technical problems including altered expression of MAPK4 in the continuously cultured LNCaP cells with stable overexpression or knockdown of MAPK4. Accordingly, we have generated LNCaP cells with Dox-inducible knockdown or Dox-inducible overexpression of MAPK4. We have also created HPS19I cells overexpressing the dominant negative TβRII. These provide key reagents for our proposed in vitro and in vivo studies. We expect to be able to finish the proposed studies at the conclusion of this award.

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Novel Therapeutic Targets to Inhibit Tumor Microenvironment Induced Castration-resistant Prostate Cancer

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Introduction

AR signaling is essential for prostate cancer (PCa) cell growth and survival. Therefore, androgen-deprivation therapy (ADT) is a standard therapy for advanced and metastatic PCa. However, most initially responsive PCa will relapse after ADT and become lethal castration-resistant PCa (CRPC). Interestingly, most CRPCs are still AR-dependent [1, 2]. Hence, AR remains as a therapeutic target for CRPC. To develop novel therapies, it is essential to delineate the underlying mechanisms for the development of CRPC and the re-activation of AR in CRPC. Tumor microenvironment plays critical roles in regulating PCa progression [3]; however, how it affects PCa AR action remains poorly understood. By using an *in vitro* PCa/stroma co-culture model and cDNA microarray analysis, we found that TGF-β signaling in prostate stroma induces the expression of several AR targets, *i.e.* PSA, TMPRSS2, and KLK4, in PCa cells in the absence of androgen. This indicates that prostate stromal TGF-β signaling induces androgen-independent AR activation in PCa. In addition, we found that stromal TGF-β signaling strongly induces MAPK4 expression in PCa. MAPK4 is an atypical MAPK not well studied [4-6]. Its biological role in cancer remains unknown. We showed that MAPK4 overexpression in PCa strongly activates AR independent of androgen, and MAPK4 knockdown inhibits AR activation. These suggest that MAPK4 is a key factor in inducing ligand-independent AR activation in PCa. Finally, cDNA microarray on total RNA extracted from laser-captured PCa cells from human CRPC tumors revealed that MAPK4 expression is strongly correlated with AR activation (expression of PSA) in human CRPC tissues, which strongly supports the human relevance of our studies. Based on our exciting preliminary results, we propose a novel and direct mechanism for the induction of CRPC relapse: Tumor microenvironment (such as stromal TGF-β signaling) induction of MAPK4 drives androgen-independent AR activation in PCa. This pathway to CRPC raises the prospects for novel therapeutic avenues in combination with ADT, anti-TGF-β strategies directed toward the tumor microenvironment, and anti-MAPK4 therapy directed to the tumor itself. We will test our novel hypothesis by *in vitro* PCa/stroma co-culture studies, *in vivo* xenograft tumor studies, and IHC studies on human CRPC tissue arrays.

Keywords:

prostate cancer, tumor microenvironment, castration resistance, AR, TGF-β, MDV3100, prostate stroma.

Overall Project Summary

Major Task 1: Study prostate stromal TGF-β signaling induced androgen-independent AR activation in PCa and determine the biological roles of MAPK4 in mediating this action. These include Subtasks (1) Confirm that AR is required for the prostate stromal TGF-β signaling induction of PSA, KLK4, and TMPRSS2 in the TGF-β treated LNCaP/HPS19I co-cultures (1-6 months), (2) Confirm that stromal TGF-β signaling induced AR activity is ligand independent (insensitive to MDV3100 treatment) (1-6 months), and (3) Determine whether

MAPK4 is required for the prostate stromal TGF-β signaling induced AR activation in LNCaP cells (6-12 months).

For Subtask 1 and Subtask 2, we have finished the proposed studies as described in our previous annual report and in our publication that was also included in our previous annual report (Yang et al, Oncotarget, 2014) [7].

For Subtask 3, we proposed to determine whether MAPK4 is required for the prostate stromal TGF-β signaling induced AR activation in LNCaP cells. To this end, we have generated the LNCaP cells with stable knockdown of MAPK4 (Figure 1). Consistent with our previous data in VCaP cells, knockdown of MAPK4 led to inhibition of AR, as well as GATA2, a transcription factor / pioneer factor critical for AR expression and activation [8]. Our data on knockdown of GATA2 using siRNA approaches also supported a critical role of GATA2 in AR expression in LNCaP cells (Figure 2). These data along with other data not presented here indicate that MAPK4 may induce AR expression/activation through a GATA2-dependent mechanism. We noticed that continuous passaging cells with stable knockdown of MAPK4 led to decreased knockdown efficiency due to the growth disadvantage of those cells with high knockdown efficiency. To prevent this, we created LNCaP cells with Dox inducible knockdown of MAPK4 using pInducer10 lentiviral inducible RNA interference system [9]. However, this inducible system requires high concentration of Dox (such as 4 μg/ml), which we later found out showed significant toxicity to LNCaP cells (data not shown). Accordingly, we have sub-cloned our shRNA coding sequences into the pLKO-tet-on system, which typically requires low concentration of Dox for inducible knockdown. We are in the process of characterizing these newly created LNCaP cells. Once we confirm the induced knockdown of MAPK4 in these cells, we will set up the LNCaP/HPS19I co-cultures and investigate whether knockdown of MAPK4 inhibits stromal TGF-β signaling induced AR activation. We will also use these engineered cells for the xenograft studies in the Subtask 3 of Major Task 2, as described below in detail.
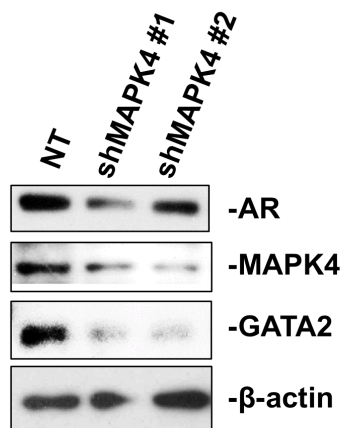

Figure 1. Stable knockdown of MAPK4 in LNCaP cells at early passage (passage #2). LNCaP cells were infected with pGIPZ lentiviruses expressing shRNA against MAPK4 or a non-targeting control (NT). After puromycin selection and one additional passage, the cell lysates were prepared and applied in Western blots for AR, MAPK4, GATA2 and β-actin. NT: non-targeting control. shMAPK4 #1 and shMAPK4 #2: two independent shRNAs against MAPK4.

Major Task 2: Determine whether inhibition of MAPK4 (in PCa) and TGF-β signaling (in stroma) enhances castration response of PCa xenografts. These include Subtasks (1). Compare LNCaP-MAPK4 *vs.* LNCaP-Ctrl xenograft growth and their response to castration (8-24 months), (2) Optimize experimental conditions for LNCaP/HPS19I xenograft studies to produce tumors with consistent HPS19I content and tumor size at the time for castration (12-24
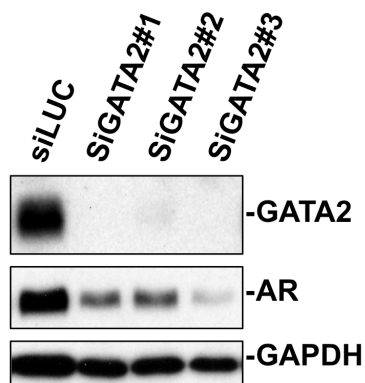
Figure 2. Knockdown of GATA2 greatly inhibits AR expression in LNCaP cells. LNCaP cells were transfected with three independent siRNAs against GATA2 (siGATA2) and siRNAs against luciferase (siLuc, as negative control). 3 days later, the cell lysates were prepared and applied in Western blots for GATA2, AR and GAPDH.

months), and (3) Compare tumor growth in intact mice and their response to castration among xenografts made with LNCaP cells (knockdown of MAPK4 vs. control) and HPS19I cells (overexpression of dominant negative TβRII vs. control) (8-36 months).

For Subtask 1, as previously discussed in our previous Annual Report, we had observed that extensive culture of LNCaP-MAPK4 cells somehow led to significantly reduced or loss of MAPK4 overexpression in these cells. Therefore, in this second year, we have been trying to generate fresh LNCaP cells overexpressing MAPK4, and directly use them in the xenograft studies. However, we have not been able to overexpress MAPK4 at high levels without going through cell sorting (GFP+, data not shown), and it is a technically difficult to obtain enough fresh cells from cell sorting to be directly used in xenograft studies. In addition, LNCaP xenograft studies routinely take over a month, especially when castration procedures are included. This itself represents a long period for potential significantly reduced or loss of MAPK4 overexpression in these LNCaP xenografts, which makes it difficult to interpret the data. Therefore, we have explored using an alternative approach by generating LNCaP cells overexpressing MAPK4 in a Dox-inducible manner using pInducer20 lentiviral gene expression system [9]. As shown in Figure 3, we have successfully generated such LNCaP cells (named as LNCaP-inMAPK4 for inducible expression of MAPK4), and demonstrated that 5-day induction using low concentration of Dox (0.5 μg/ml) is enough to induce MAPK4 expression, which is accompanied with increased AR expression. However, qRT-PCR analysis reveals minimal levels of AR activation (induction of PSA expression). We suspect that the MAPK4 overexpression has not reached the threshold (such as levels comparable to that of VCaP cells), which can robustly enhance AR activation. To enrich LNCaP cells highly overexpressing
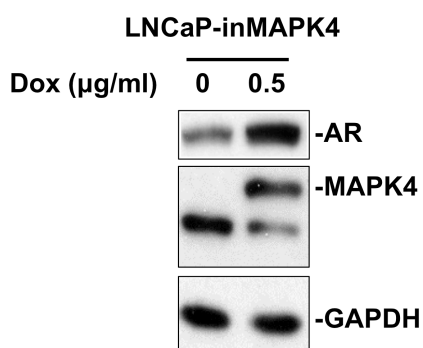

Figure 3. LNCaP-inMAPK4 cells were treated with 0.5 μg/ml of Dox or vehicle control for 5 days. The cell lysates were then prepared and applied in Western blot assays. 0.5 μg/ml of Dox significantly induces MAPK4 expression in these cells. AR expression is induced in the Dox-induced MAPK4 overexpressing cells.

MAPK4, we will perform cell sorting (GFP) on Dox (0.5 µg/ml, >5 days) treated LNCaP-inMAPK4 cells. We will sort out the top GFP/MAPK4 expressors, and expand them in the absence of Dox induction. This will produce LNCaP-inMAPK4 cells expressing high levels of MAPK4 upon Dox induction. Once we confirm this, we will use these LNCaP-inMAPK4 cells and/or LNCaP-inCtrl cells (control cells after cell sorting, if needed) for the proposed xenograft studies in Subtask 1. In this case, we may begin inducing MAPK4 expression in the xenografts (adding Dox into drinking water) when they reach certain sizes and/or shortly before surgical castration to determine MAPK4 effects on tumor growth, castration response and/or development of castration-resistant prostate cancer.

For Subtask 2, we have performed pilot studies to test the ratio of LNCaP vs. HPS19I cells at the time of xenograft inoculation and found that a ratio ranging from 2:1 to 4:1 produces tumors with consistent HPS19I content and tumor size at the time for castration.

For Subtask 3, we have engineered HPS19I cells to overexpress a dominant negative TβRII (dnTβRII) vs. control for the proposed xenograft studies. To do this, we first used the pLNCX-dnTβRII retroviral gene expressing vector [10] to engineer HPS19I cells for dnTβRII overexpression. However, we found that it is difficult to infect the HPS19I cells using this method and the pLNCX-dnTβRII vector produced low dnTβRII expression when transiently transfected into 293T cells (Figure 4). To enhance dnTβRII expression and infection efficiency, we have PCR amplified the dnTβRII cDNA from the pLNCX-dnTβRII vector and cloned it into the pCDH lentiviral vector (pCDH-dnTβRII). We have successfully engineered HPS19I cells for dnTβRII overexpression and 100% of the cells are RFP+ (indication for dnTβRII expression) after selection using 0.7 µg/ml of puromycin. However, we observed that the engineered HPS19I cells, which themselves are slow-growing cells, grew extremely slowly and eventually died out when cultured in 0.7 µg/ml of puromycin. Accordingly, we have re-engineered these cells and are now expanding these cells in the absence of puromycin after one week of selection using 0.7 µg/ml of puromycin.   As similarly described in Task 1, we are also in the process of generating and characterizing the LNCaP cells with inducible knockdown of MAPK4. Once we obtain these cell lines, we will carry out the proposed studies in full-scale.
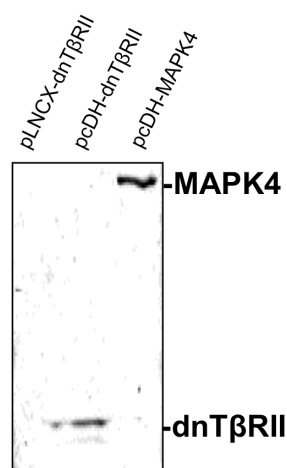

Figure 4. The expression of the Flag-tagged dnTβRII and the Flag-tagged MAPK4 in transiently transfected 293T cells. The pLNCX-dnTβRII, pCDH-dnTβRII and pCDH-MAPK4 vectors were transiently transfected into 293T cells. 3 days later, the cell lysates were prepared and applied in Western blots using an anti-Flag antibody.

Major Task 3: Determine whether MAPK4 expression correlates with AR expression / activation in PCa cells and TGF-β signaling activation in stromal cells in human CRPC tissues. These include Subtasks (1) Perform qRT-PCR on cDNA from human CRPC tissues for the expression of MAPK4, AR, PSA, KLK4, COL1A1, FAP, and K-Alpha-1 (18-36 months), and (2) Optimize IHC protocols and perform IHC studies on human PCa tissue-microarray slides for the expression of MAPK4, AR, PSA, and phospho-Smad2 (18-36 months).

The proposed studies need approval from both local IRB and HRPO. We have submitted the proposed studies to the local IRB for review and received a decision of Non-Human Subject Research (NHSR) status. We have submitted the HRPO application form along with our Human Protocol Report to local IRB and the NHSR decision letter for review by HRPO. This application is now pending. Therefore, we have not begun the proposed studies in Major Task 3.

Key Research Accomplishments

- Submission of a revised manuscript describing part of this funded study (Yang et al, Oncotarget, 2014) [7].

- Received a decision of Non-Human Subject Research (NHSR) status for the proposed studies in Tasks 3 from local IRB.

- Optimization of experimental conditions for the LNCaP/HPS19I xenograft studies to produce tumors with consistent HPS19I content and tumor size at the time for castration.

- Generation of the LNCaP cells with Dox-inducible knockdown of MAPK4 based on both the pInducer10 and the pLKO-tet-on system.

- Generation of LNCaP cells with Dox-inducible overexpression of MAPK4 based on the pInducer20 lentiviral gene expression system.

- Generation of the pCDH-dnTβRII lentiviral vector, based on which the HPS19I cells overexpressing the dnTβRII were generated.

Conclusion

In this second year (Sept. 15, 2014 - Sept. 14, 2015), as continuation of our work in the first year, we finished all the experiments suggested by the reviewers and submitted a revised manuscript to Oncotarget on Sept. 25, 2014, which was later published on Oct. 14 (Yang et al, Oncotarget, 2014) [7]. Please note, this paper was also included as part of the first annual report since it was initially submitted in the first year of funding.

Due to technical problems, including the altered expression of MAPK4 in the continuously cultured LNCaP cells with stable overexpression or knockdown of MAPK4, we have not been able to carry out full-scale xenograft studies in Subtask 1 and 3 of Major task 2.

However, we have performed and/or finished trouble shooting and generated key cell lines including the LNCaP cells with Dox-inducible overexpression of MAPK4 and with Dox-inducible knockdown of MAPK4, as well as the HPS19I cells overexpressing dnTβII. Similarly, we will perform the co-cultures in the Subtask 3 of Major task 1 using the newly created LNCaP cells with Dox-inducible knockdown of MAPK4 for more consistent results. We will begin studies in Major Task 3 after receiving approval from HRPO. All in all, although delayed, we expect to be able to finish the proposed studies at the conclusion of the award.

Publications, Abstracts, and Presentations:

1. Yang F*, Chen Y, Shen T, Guo D, Dakhova O, Ittmann MM, Creighton CJ, Zhang Y, Dang TD, Rowley DR*. Stromal TGF-β signaling induces AR activation in prostate cancer. **Oncotarget**. 2014 Nov 15;5(21):10854-69.

Please note that this paper was revised and accepted for publication in this second year of funding. It was also reported in our first Annual Report since it was initially submitted in the first year. It is not included in the Appendices to avoid duplication.

Inventions, patents and licenses: Nothing to report

Reportable Outcomes: Nothing to report

Other Achievements: Nothing to report

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Appendices: None.